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Self-Complementary Adeno-associated Virus Serotype 2 Vector: Global Distribution and Broad Dispersion of AAV-Mediated Transgene Expression in Mouse Brain

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The blood–brain barrier is the main obstacle to efficient delivery of therapeutic reagents, including viral vectors, into the central nervous system (CNS) for treating global CNS diseases. In this study, the effects of mannitol infusions on global brain gene expression of a novel AAV vector were examined after intravenous (iv) or intracisternal injection. Initially, a self-complementary adeno-associated virus serotype 2 vector (scAAV) was compared to traditional single-stranded AAV2 vector for reporter gene expression in the brain of adult mice with or without pretreatment of an iv mannitol infusion. One to two months postinjection, analysis of vector-transduced green fluorescent protein (GFP) expression in the brain revealed that vector delivery to the CNS via iv injection required pretreatment with mannitol. This expression was observed only when scAAV vectors were used. Using these conditions, transgene expression was observed in various neurons and glial cells throughout the brain. The peripherally administered scAAV vectors also transduced the cells in multiple somatic tissues with efficient expression in liver (20–30% of hepatocytes), but was less efficient in other somatic tissues. Intracisternal injection of scAAV vector produced a broad and intense transgene expression in both neurons and glial cells in the CNS of injected mice ranging from the olfactory area to the brain stem and spinal cord. More than 50% of the Purkinje cells in the cerebellum expressed GFP. Intravenous infusion of mannitol before intracisternal injection of the scAAV vector enhanced the dispersion of the vector in the CNS. Further optimization of these steps combining peripheral and intracisternal scAAV gene delivery should facilitate the development of treatments for global CNS diseases, especially diseases involving both the somatic system and the CNS, such as lysosomal storage disorders.

INTRODUCTION

Adeno-associated virus (AAV) is a widespread parvovirus with no known pathogenesis in human [1] and is known to infect a wide range of human tissues. The recombinant AAV viral vector system [2], containing less than 4% of viral genome, has been proven to transduce various cell types, including both neuronal and nonneuronal cells in the mammalian central nervous system (CNS), following direct injection into the brain [3–8]. Conventional recombinant AAV vectors deliver a single-stranded DNA genome, which must be converted by host-cell-mediated DNA synthesis to double-stranded DNA for active expression. At high multiplicities of infection, hybridization of complementary DNA strands from separate virions may also generate active double-stranded templates for expression [9]. This requirement for formation of duplex DNA has proven to be an important limiting factor for AAV vector transduction. The recently developed self-complementary AAV (scAAV) vector system provides an invaluable tool in AAV-mediated gene therapy studies for CNS diseases due to its high transduction efficiency [10]. The scAAV vector is made from constructs in which the replicating DNA is less than half the length of the wtAAV genome, thus allowing inverted repeat dimer molecules to be encapsidated but limiting the size of the transgene. The two halves of these molecules are complementary to each other, and base-pair into a double-stranded hairpin molecule once released from the capsid. Because the kinetics of this reaction are first order with respect to the number of DNA molecules involved, the efficiency of transduction (i.e., transducing units per genome-contain-

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ing particle) is independent of multiplicity of infection and host-cell DNA synthesis

The blood–brain barrier (BBB) plays an important role in preventing microorganisms and large molecules from entering the CNS [11]. Likewise, it is also a major obstacle in developing new therapies for CNS diseases because it impedes effective delivery of therapeutic reagents, including AAV vectors, into the CNS. Many studies have been carried out to interrupt the BBB or have used escort systems, with the ultimate goal of enabling peripherally delivered therapeutic substances to cross the BBB [12–17].

Mannitol is a known blood–brain barrier interruptive reagent. Intravenous infusion of highly concentrated mannitol (25%) is routinely used to reduce the intracranial pressure in patients with traumatic brain diseases, by pulling fluid from the CNS by temporarily increasing vascular osmotic pressure. Many human clinical studies have been performed with intra-arterial (carotid) infusion of mannitol or other sugar solution, to open the BBB to enhance the CNS delivery of chemotherapeutic reagents. Improved survival in patients with brain tumors has been demonstrated [12,14]. Mannitol has been used in preclinical studies to achieve the entrance of a wide range of substances into brain, including enzymes, antibodies, and viral vectors [12,18,19]. Intra-arterial infusion of mannitol caused no obvious BBB damage [20].

In this study, we have used a self-complementary AAV2 vector and mannitol infusion to develop methods of peripheral or intracisternal vector delivery, which resulted in broad dispersion of AAV-mediated transgene expression in mouse CNS and in multiple somatic tissues/organs.

RESULTS

AAV-Mediated Transgene Expression in Mouse Brain after Direct Microinjection

We compared the distribution of transgene expression in adult mouse brain after direct injection of scAAV2 or single-stranded (ss) AAV2 vectors into the thalamus or dorsal third ventricle. Four weeks after injection, both scAAV2 vector and ssAAV2 vector were shown to transduce both neuronal and nonneuronal cells. Again, while we observed no obvious difference in the distribution of transgene expression in brain, green fluorescent protein (GFP) expression from the scAAV2 vector was significantly more intense, and involved a greater number of cells, than that from the ssAAV2 vector (Fig. 1).

Global Distribution of AAV-Mediated Transgene Expression in Mouse Brain after Systematic Vector Delivery: Influence of Mannitol

In an attempt to facilitate delivery of AAV vector into the CNS of adult mice via peripheral administration, we injected the scAAV2 viral vector intravenously into adult mice 10–20 min after an iv infusion of 25% mannitol to disrupt the blood–brain barrier. Four to eight weeks after injection, we carried out fluorescence microscopy to visualize the AAV-mediated transgene expression on serial Vibratome brain sections.

The iv injection of the scAAV2 vectors (4×10^{11} viral particles) produced a global distribution of GFP expression in the CNS (brain and spinal cord) of all 16 mice only when it was preceded by an iv administration of 25% mannitol (Fig. 2). We observed no GFP expression in the CNS of the mice injected iv with only the scAAV2-CMV-GFP viral vector or with only the scAAV2-CMV-GFP viral vector in 12.5% mannitol. We did not see GFP in the brain of the mice given an iv injection of scAAV2-CMV-GFP vectors immediately after the injection of mannitol. We observed no transgene expression in the brains of the mice receiving an iv injection of conventional single-stranded AAV2 viral vector after iv injection with mannitol (25%).

In the mice that received the pretreatment with 25% mannitol, we observed GFP expression from the scAAV2 vector in both neuronal and nonneuronal cells (Fig. 2), including various neurons, some glial cells, and cells of the choroid plexus. The transduced neurons included Purkinje cells, granule cells, and stellate cells of cerebellum and neurons in cerebral cortex, hippocampus, thalamus, hypothalamus, brain stem, and the olfactory area. The intensity of GFP expression varied among cells. The GFP-expressing cells were distributed throughout the brain of the injected mouse, although nearly 50% of them were seen in cerebellum and brain stem. The number of brain cells observed expressing GFP in each mouse brain was 47–58 when 4×10^{11} viral particles were injected intravenously 20 min after the iv administration of mannitol. The GFP-expressing cells increased to 58–132 when intravenous injection of the vector was conducted at 15 min after iv infusion of mannitol and to over 500 when the vector was delivered at 10 min after mannitol was administered.

Broad Dispersion of AAV Transgene Expression in the Brain after Intracisternal Injection of scAAV2 Vector

We carried out intracisternal injection of scAAV vector in 4- to 6-week-old normal mice to study the efficiency of CNS vector delivery with this approach. We compared the AAV-mediated GFP gene expression in mouse brains in the intracisternally injected mice with ($n = 8$) or without ($n = 4$) prior iv infusion of mannitol, to determine whether dispersion of the AAV vector would be affected.

Intracisternal delivery resulted in a broad spread of the scAAV2 vector in mouse CNS (Fig. 3). We observed broader dispersion and more intense GFP expression in the CNS of the mice infused iv with mannitol 15–20 min before intracisternal injection of AAV vector, compared to that in the CNS of the mice that were not pretreated (Fig. 3). In the mice intracisternally injected with scAAV2 vector, we observed transgene expression in neuronal and

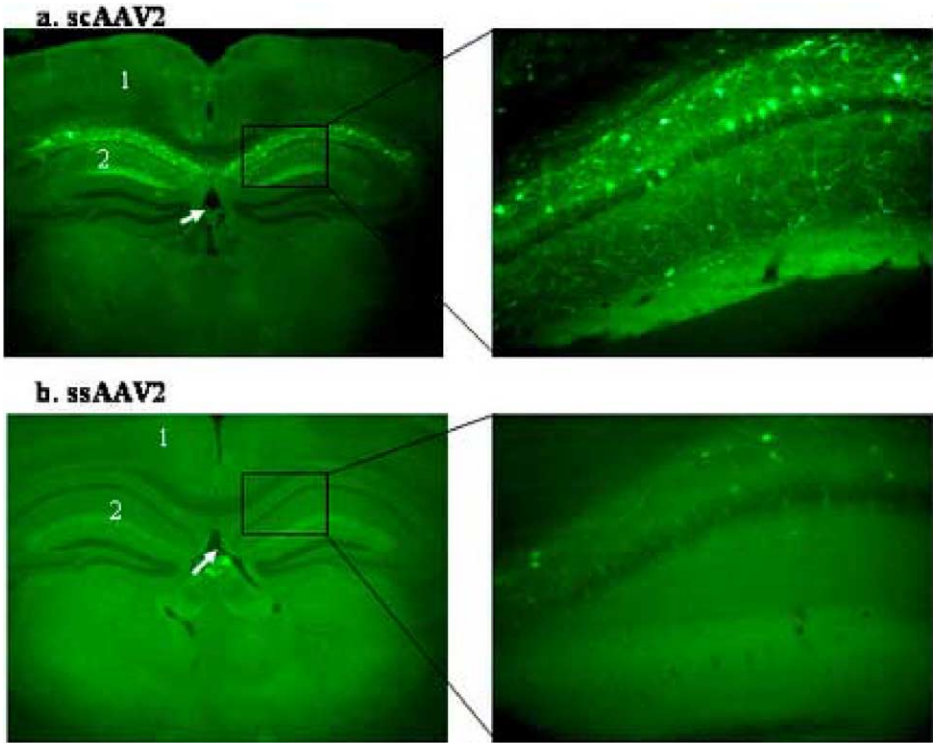

FIG. 1. Comparison of AAV-mediated transgene expression in mouse brain by direct injection. Self-complementary AAV2 vector scAAV-CMV-GFP and conventional AAV2 vector ssAAV-CMV-GFP (1×10^8/μl) were delivered into the dorsal third ventricle (5 μl/injection/mouse) of normal adult mice (2–3 months of age) by direct microinjection. Transverse Vibratome sections of whole brain sample were analyzed 4 weeks after infusion. Sections at bregma −1.58. (a) scAAV2: self-complementary AAV2 vector. (b) ssAAV2: conventional AAV2 vector. 1, Cerebral cortex; 2, hippocampus; arrow, dorsal third ventricle.

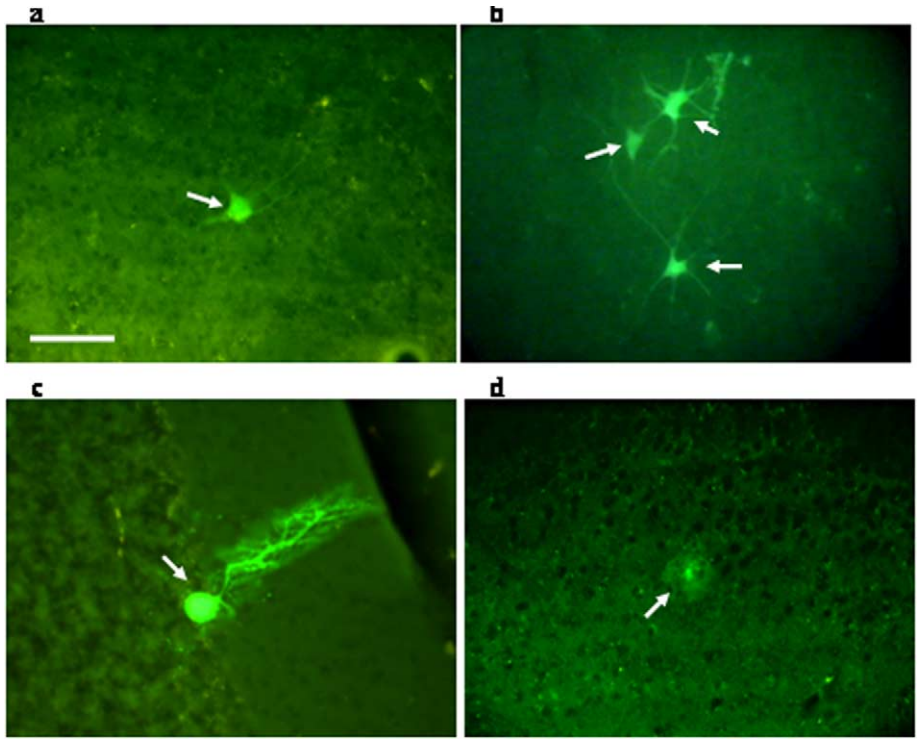

FIG. 2. AAV-mediated transgene expression in mouse brain after intravenous injection. Self-complementary AAV2 vectors (4×10^{11} in 200 μl PBS) were delivered into adult mice (2–3 months of age) after an iv infusion of 200 μl mannitol (25%). Four or eight weeks after infusion, multiple tissue samples were collected after transcardial perfusion. Transverse sections (50 μm) of entire brain were obtained using a Vibratome for fluorescence microscopy. (a) A neuron in the thalamus. (b) Neurons in brain stem. (c) A Purkinje cell in the cerebellum. (d) A glial cell in cerebral cortex (see arrows). Bar, 100 μm.

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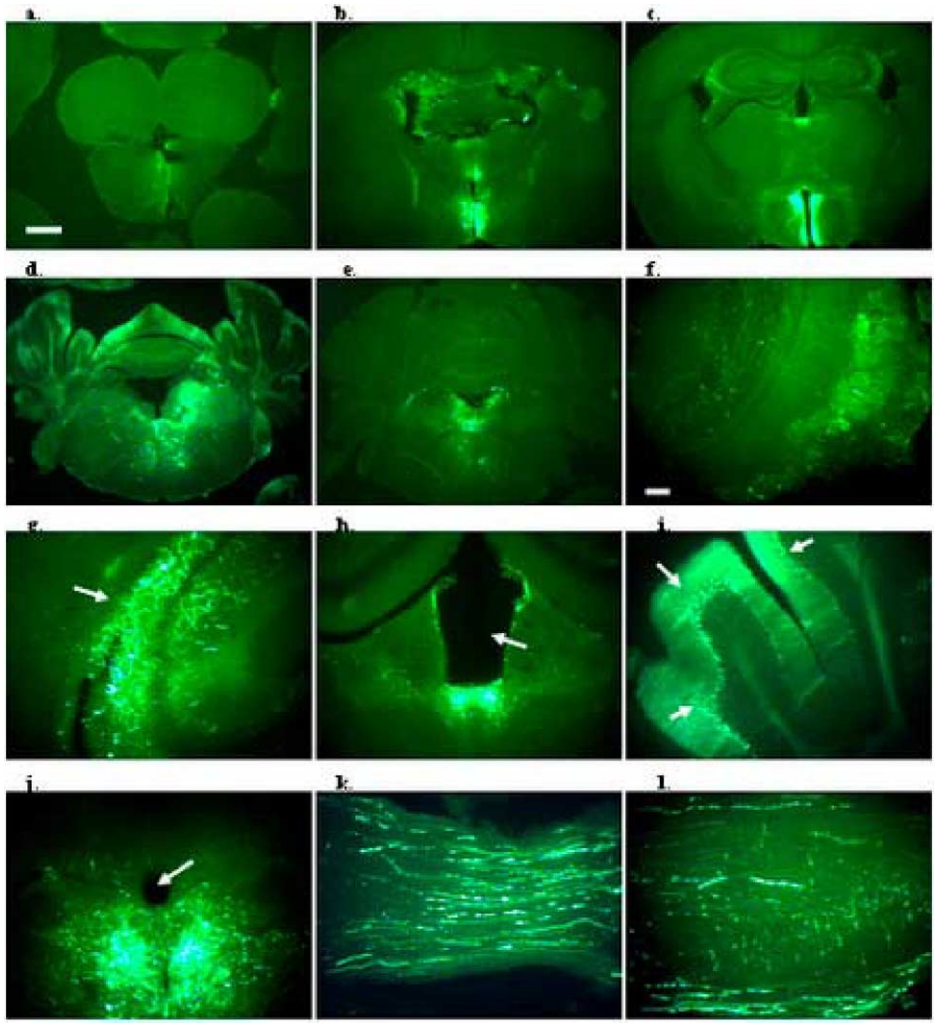

FIG. 3. AAV-mediated GFP expression in mouse brain after intracisternal injection. Self-complementary AAV2 vector (4×10^{10} viral particles in 15 μl PBS) was delivered into the posterior cistern of mice (4–6 weeks of age) 20 min after an iv infusion of 200 μl mannitol (25%). Transverse sections (50 μm) of whole brain were prepared for fluorescence microscopy 4 weeks after infusion, using the stereotaxic coordinates of Franklin and Paxinos. (a–e) Bar, 1.0 mm. (f–l) Bar, 100 μm. (a) Bregma 3.08 mm, (b) bregma −0.34 mm, (c) bregma −1.34 mm, (d) bregma −5.52 mm. (e) A brain section (bregma −5.68 mm) of a mouse intracisternally injected with scAAV 2 vector, without pretreatment with mannitol. (f) Bregma 2.80, olfactory bulb; (g) bregma −1.82, arrow—hippocampus; (h) bregma −1.34, arrow—dorsal third ventricle; (i) cerebellum, arrows—Purkinje cells; (j) brain stem, arrow—fourth ventricle (bregma −5.40); (k) cervical spinal cord (ventral white matter, longitudinal section). l, Cervical spinal cord (half longitudinal section); bottom, lateral surface; top, middle line.

nonneuronal cells in many areas throughout the mouse brain, including olfactory, hippocampus, striatum, corpus callosum, thalamus, hypothalamus, cerebral cortex, cerebella, and brain stem (Fig. 3). We saw more intensive GFP expression in cerebellum and brain stem, compared to that in the other brain areas. Purkinje cells (more than 50%) and small cells in the molecular layer and granule layer were efficiently transduced (Fig. 3). We observed

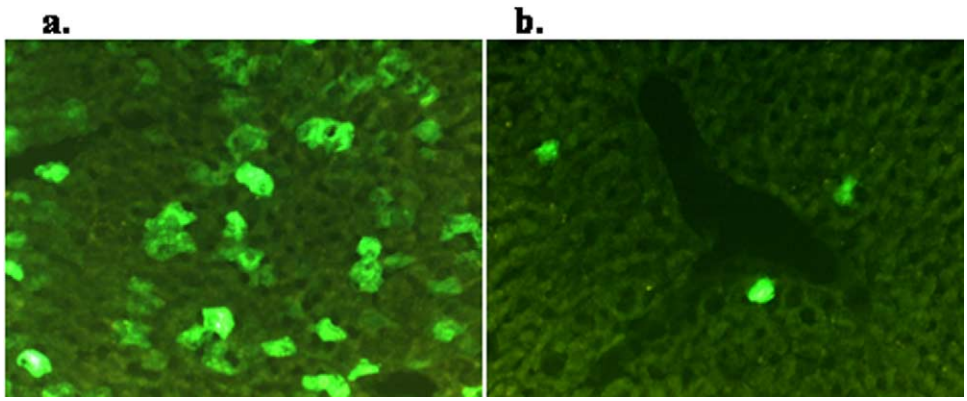

FIG. 4. AAV-mediated transduction in mouse liver by intravenous delivery. Self-complementary AAV2 vectors (scAAV-CMV-GFP) or conventional AAV vectors (ssAAV-CMV-GFP) (4×10^{11} in 200 μl PBS) were delivered into adult mice (2–3 months of age) 20–30 min after an iv infusion of 200 μl mannitol (25%). Cryostat sections (30 μm) of liver samples were analyzed for GFP expression 8 weeks after infusion. (a) scAAV2: self-complementary AAV 2 vectors. (b) ssAAV2: conventional AAV vectors.

GFP-positive projections throughout the brain and spinal cord (Fig. 3). More cells were seen to express GFP in the areas with cerebral–spinal fluid contact, i.e., the cells surrounding the ventricle system. Only sporadic cells, in cerebral cortex and spinal cord, expressed GFP. We observed no obvious sign of toxicological reaction or cell damage in the brains of injected mice and the animals appeared healthy during the period of the experiments.

Intracisternal injection of ssAAV vector resulted in a similar distribution of GFP expression in mouse brains but the intensity of the transgene expression was much lower compared to that mediated by scAAV vector (Fig. 3e).

AAV-Mediated Transduction in Peripheral Tissues after iv Administration

We also surveyed the distribution of AAV-mediated transduction in multiple somatic tissues of mice, with or without the iv injection of mannitol. We observed no obvious difference in the number of cells transduced, or in the intensity of GFP expression, in somatic tissues with or without mannitol pretreatment.

In the livers of mice injected iv with 4×10^{11} vector particles of scAAV2-CMV-GFP, we observed transgene expression in 20–30% of hepatocytes (Fig. 4). We saw lower numbers of GFP-positive cells in other somatic tissues: $<3\%$ in heart, lung, adipose tissue, and spleen; $<1\%$ in intestine and skeletal muscle (data not shown); and only occasionally in kidney. Intravenous injection of the ssAAV vectors resulted in visible GFP expression in only 1–2% of hepatocytes in liver (Fig. 4), but not in other tissues. We observed no apparent cell damage in the tissues of animals injected with either vector.

Discussion

A recently developed scAAV vector, carrying a dimeric genome, has a much higher transduction efficiency than the conventional rAAV vector, which contains only a single-copy, single-stranded genome [10]. We have taken advantage of the transduction potency of the scAAV viral vector system to map and optimize vector distribution in the CNS and peripheral tissues. Because of this greater sensitivity we were able to demonstrate that pretreatment with an iv infusion of mannitol facilitated entry of peripherally delivered AAV vector into the CNS, by presumably interrupting the blood–brain barrier. Intracisternal administration of AAV vector resulted in broad distribution of transgene expression in the mouse CNS, which was enhanced by iv infusion of mannitol prior to the vector injection.

Using the scAAV2 viral vector, a global distribution of AAV-mediated transgene expression was achieved in mouse brain by systemic delivery of the vectors into animals pretreated with an intravenous infusion of 25% of mannitol. This was not observed using ssAAV vectors, thus illustrating the utility of scAAV in biodistribution studies. Because these vectors are transduced independent of cellular DNA synthesis, the scAAV vectors are not limited by the ability of the recipient cell to replicate DNA or convert single-stranded AAV genome to duplex or by the stability of the single-stranded genomes. While this may not translate directly to the efficiency of transduction ultimately achieved with the ssAAV vector, parameters such as cell and tissue tropism using different AAV serotypes or methodologies of vector delivery can be reliably established.

Previous studies have shown the access of substances to the CNS in human and experimental animals after intra-arterial administration of mannitol [12]. This study demonstrated that an iv infusion of mannitol disrupted the blood–brain barrier to a degree that allowed peripherally delivered scAAV viral vectors to enter the CNS and transduce both neuronal and nonneuronal cells throughout the brain. However, the disruption of the blood–brain barrier by mannitol is temporary and reversible, and the BBB can rapidly regain its integrity through the physiological rearrangement of the body fluid. Therefore, the timing of AAV vector delivery after the infusion of mannitol is critical to the efficiency of the CNS entry. The optimal timing is unclear for opening of the BBB, but the clinical effects of mannitol occur typically within 20–30 min of iv injection in humans. Previous studies showed that by intra-arterial administration of mannitol through the carotid artery, the peak opening of the BBB was 5 min after infusion [27] and the opening lasted for 20–30 min [12]. This study showed a 10-fold increase in the number of the transgene expressing cells, when the time between the iv infusion of AAV vector and the iv administration of mannitol was decreased from 20 to 10 min. Additional time-course studies will be needed to determine the optimal window for iv injection of AAV vectors, after iv infusion of mannitol, to achieve the highest level of transduction in the CNS by systemic delivery.

Using intracisternal injection of the scAAV2 vector, this study demonstrated a high efficiency and broad distribution of transgene expression in mouse CNS, an effect that was enhanced by systemic mannitol administration. The pretreatment with mannitol may facilitate the diffusion of the vectors intrastitially through brain tissue and/or increase the entry into cells, due to the relative dehydration of the brain by mannitol. The intracisternally delivered AAV vectors appeared to spread extensively in the CNS, mainly through the circulation of cerebral spinal fluid (CSF). This route of administration may therefore provide a great potential for AAV-mediated CNS gene delivery. In addition, intracisternal injection allows us to deliver the vectors safely to mouse CNS in a relatively large volume, compared to intraventricular injection, since the physical characteristics of the injection site provide higher buffer capacity for the pressure increase in the system. Intracisternal injection may thus contribute significantly to the development of AAV gene therapies

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for global CNS diseases. The translation of this technique to human clinical trials at this time may be limited, since the volume of the intracisternal injection was about 25% of the total volume of CSF in adult mice. Although the use of mannitol may reduce the intracranial volume and possibly improve vector delivery, feasibility studies in small and large animals are needed prior to considering clinical trials in humans.

Finally, this study also demonstrated a widespread transgene expression in the peripheral system after iv infusion with AAV, although differences in transduction efficiency among organs were observed. These differences may be due to many factors, including the abundance of blood supply to the organ, the level of AAV2 receptor expression on the cell surfaces in different tissues, different subcellular trafficking pathways in the different cell types, or differential activity of the cytomegalovirus (CMV) promoter in each cell type. Importantly, in contrast to the brain tissue, there was no obvious difference in the transduction efficiency in somatic tissues of mice in the presence or absence of mannitol. We therefore anticipate that administration of mannitol has little or no direct effect on the efficiency of AAV-mediated transduction in peripheral tissues. The wide tropism of AAV2 to different organs and tissues after iv injection suggests the potential of AAV gene delivery for diseases involving multiple organs/tissues, such as lysosomal storage disorders. Such treatments may be further enhanced through multiple applications of different AAV serotypes carrying the same transgene, thus maximizing the numbers and types of cells genetically corrected. Since intracisternal administration leads to wide dispersion of AAV-mediated transduction through the CNS, combining intravenous and intracisternal delivery of AAV vector with mannitol pretreatment could significantly enhance the therapeutic effects for diseases with both CNS and somatic involvement.

MATERIALS AND METHODS

AAV viral vectors. A recently constructed AAV2 vector plasmid, pHpa-Trs-SK (manuscript in preparation), was used to produce the self-complementary AAV2 viral vector (scAAV2-CMV-GFP) containing a human CMV promoter and an enhanced GFP gene. This construct produces only dimeric inverted repeat genomes during replication since the terminal resolution site is deleted from one of the terminal repeats, inactivating it as an origin of replication. Vectors prepared using this mutant background contain greater than 90% scAAV vector genomes. A control AAV2 vector plasmid, pTRUF-GFP, was used to produce the conventional AAV2 viral vector (ssAAV-CMV-GFP), also expressing GFP and driven by a CMV promoter.

The recombinant AAV2 viral vectors were produced in 293 cells using three-plasmid cotransfection, and purified following previously published methods [21,22] by the Vector Core Facility, Gene Therapy Center, University of North Carolina at Chapel Hill.

Animals. Normal C57 mice (2–3 months of age) used in this study were bred and maintained in the Laboratory Animal Facility of the University of North Carolina at Chapel Hill. All the procedures were approved by IACUC at the university. All care and procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* (NHHS Publication No. (NIH) 85-23).

Direct microinjection of AAV vectors into mouse brain. scAAV2-CMV-GFP and ssAAV2-CMV-GFP viral vectors were delivered into the thalamus or the dorsal third ventricle of normal mice, 2–3 months of age, by direct microinjection. The stereotaxic (KOPF) microinjection was carried out following previously published techniques [3,23], using coordinates from the mouse brain atlas of Franklin and Paxinos [24]. The mice were anesthetized with 2.5% Avertin (0.38–0.43 mg/g body weight) before the infusion. Two microliters of AAV viral vectors (1×10^8viral particles/μl) was infused into the right thalamus at a rate of 0.1 μl/min and 5 μl into the dorsal third ventricle at a rate of 0.5 μl/min, one injection per site per mouse, four mice per experimental group.

Four weeks after infusion, the animals were anesthetized with 2.5% Avertin and then perfused transcardially with cold 0.1 M phosphate-buffered saline (PBS) (pH 7.4), followed by 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). The entire mouse brain samples were collected and fixed at 4°C in 4% paraformaldehyde for fluorescence microscopy.

Intracisternal injection. Intracisternal injection was conducted in normal mice (4–6 weeks of age), four mice per experimental group, following previously published procedures [25,26]. The animals were anesthetized with intraperitoneal injection of 2.5% Avertin (0.38–0.43 mg/g body weight). Fifteen microliters of scAAV or ssAAV vector, 3×10^{10} viral particles in PBS, was rapidly injected (<2 s) into the posterior cistern of each mouse, 15–20 min after an iv infusion (<2 min) of 200 μl mannitol (25%) or without pretreatment with mannitol.

The mice were perfused transcardially as described above, 4 weeks postinjection. The brain and spinal cord samples were collected and fixed in 4% paraformaldehyde at 4°C for fluorescence microscopy.

Peripheral delivery of AAV viral vectors. AAV vectors, 4×10^{11} in PBS (pH 7.4), were delivered into adult male mice (2–3 months of age) by intravenous injection through the tail vein. The volume of each injection was 200 μl (about 10 μl/g body weight) over a period of 2 min.

The injection was carried out as follows. The animals were anesthetized by intraperitoneal injection of 2.5% Avertin (0.38–0.43 mg/g body weight) before iv infusion of a dose (200 μl) of mannitol (25%, for iv use only, Abbott Laboratories, NDC0074-4031-01) through the tail vein into each mouse over a period of 2 min. Ten to twenty minutes after the injection of mannitol, each mouse was given AAV2 vectors in 200 μl of PBS intravenously.

Controls included the following mice: iv injected with only the self-complementary AAV2 vectors, iv injected with scAAV vectors immediately after injection of a dose of mannitol, iv injected with mannitol followed by conventional AAV2 vectors, and iv injection of the scAAV2 vector in 12.5% mannitol and uninjected animals. Each experimental group included two to four mice.

Four or eight weeks after the injection, the animals were perfused transcardially as described above. The entire brain, as well as multiple somatic tissue samples, including liver, kidney, spleen, heart, lung, intestine, skeletal muscle, and fat tissue, were collected and fixed in 4% paraformaldehyde at 4°C for fluorescence microscopy.

Fluorescence microscopy. Fluorescence microscopy was carried out to visualize the AAV-mediated transgene expression. After being fixed in 4% paraformaldehyde at 4°C overnight, serial transverse brain sections (50 μm, 150–180 sections/brain) and longitudinal spinal cord sections (50 μm) were obtained using a Vibratome. Other tissue samples were embedded in OCT compound and frozen on dry ice before being sectioned (30 μm) with a cryostat. The sections were then mounted on slides and visualized with a fluorescence microscope.

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